



Consommation
et Corporations Canada

Consumer and
Corporate Affairs Canada (21) (A1)

2,011,768

Bureau des brevets

Patent Office (22)

1990/03/08

Ottawa, Canada
K1A 0C9

(43)

1990/09/10

(52)

150-15.5

(51) INTL.CL.⁵ C12Q-1/48

(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Solid Phase Protein Kinase Assay

(72) Sahal, Dinkar - India ;

(73) City of Hope - U.S.A. ;

(30) (US) 321,298 1989/03/10

(57) 18 Claims

50983-89

BEST AVAILABLE COPY

Notice: The specification contained herein as filed

Canada

CCA 3254 (10-89) 41

2011768

ABSTRACT

A solid phase assay of high sensitivity and efficiency for determination of the activity of tyrosine and serine/threonine specific protein kinases in crude enzyme preparations.

SOLID PHASE PROTEIN KINASE ASSAY

This invention relates to assays for protein kinases. More particularly the invention relates to a solid phase assays specific for tyrosine protein kinase (TPK).

BACKGROUND OF THE INVENTION

5 Protein kinases play an important role in signal transduction mechanisms regulating a variety of enzyme activities. For example, cAMP transduces the message of an external stimulus by activating the cAMP dependent Ser/Thr
10 kinases (Krebs, E.G. Biochem. Soc. Trans. 13:813-820 (1985); Lohmann, S.M., et al., Adv. Cyclic Nucleotide Res. 18:63-117 (1984)).

Membrane-bound cell surface receptors for growth factors such as insulin, insulin-like growth factor (IGF)-I and
15 epidermal growth factor initiate ligand stimulated TPK activities (Yarden, Y., et al. Biochemistry 27:3113-3119 (1988)). Association of TPK activity with oncogenes and growth factor receptors has suggested a potential involvement of tyrosine specific protein kinases in cellular oncogenesis
20 metabolism, growth, and development (Hunter, T., et al., Ann. Rev. Biochem. 54:897-930 (1985); Bishop, M. Cell 42:23-38 (1985)).

TPK is apparently much less abundant than Ser/Thr kinases in cells. High endogenous Ser/Thr phosphorylation interferes
25 with the determination of TPK activity in crude cell extracts or column chromatography eluates.

Known protein kinase assays generally require either trichloroacetic acid washing to remove excess [γ - 32 P] while keeping all phosphorylated macromolecules precipitated on filter paper (Corbin, J.D., et al. Methods in Enzymology 5 38:287-290 (1974)) or washing with mild acids such as 75 mM phosphoric acid to bind all basic protein/peptide substrates to phosphocellulose paper while anionic [γ - 32 P] ATP is washed away (Roskoski, R., Jr. Methods in Enzymology 99:3-6 (1983)).

The high Michaelis constant (K_m) requirements of TPKs for 10 peptides such as Angiotensin II or the src-related peptide have rendered peptide substrate TPK assays impractical due to high cost, notwithstanding the fact that peptides provide a better defined small molecule substrate with a specific site for TPK's than protein substrates (Casnellie, J.E., et al., Adv. Enzyme 15 Reg. 22:501-515 (1983)).

High molecular weight synthetic tyrosine-containing polypeptides are inexpensive specific substrates for a variety of TPKs. However, these polymers are disadvantageous as compared with small peptides in that trichloroacetic acid, when 20 applied to crude enzyme preparations, precipitates endogenously phosphorylated proteins along with the phosphorylated polymer substrates.

A variety of enzyme assays using polyacrylamide gels have been developed. In general, these assays separate enzyme 25 preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting gels are renatured and assayed for the enzyme activity of interest. For example, protein kinase activity of a catalytic subunit of cAMP-dependent protein kinase or an active subunit of casein 30 kinase II was determined by renaturing the dissociated enzyme in polyacrylamide gels containing 1 mg/ml casein followed by

phosphorylation with [γ - 32 P] ATP (Geahlen, R.L., et al. Anal. Biochem. 153:151-158 (1986)). After removing unreacted [γ - 32 P] ATP by washing the gel in the presence of an ion-exchange resin, the positions (Mr) of the protein kinase activity were
5 visualized by autoradiograph.

A general procedure utilizing synthetic small peptides for detecting TPK activity in crude or purified preparations is described by Glazer (Glazer, R.I., et al. Anal. Biochem. 164:214-220 (1987)). Enzyme preparations were resolved by PAGE
10 under nondenaturing conditions. The gels were incubated in [γ - 32 P] ATP-metal mixtures containing, for example, poly (Glu, Tyr) 4:1 or poly (Glu, Ala, Tyr) 6:3:1. The gels were then fixed and washed in trichloroacetic acid-pyrophosphate, dried, and analyzed by autoradiography or liquid scintillation
15 counting.

The principle of radial diffusion in substrate containing agarose gels has been applied for quantitation of proteolytic activity of various enzymes. Such assays involve the addition of the sample to wells punched in relatively thin layers of gel
20 having a substrate distributed uniformly therein. Clearing zones adjacent the wells indicate the presence of the enzyme. The diameter of the clearing zone can be quantitatively assessed with enzyme activity (See, e.g., Schumacher, G.F.B., et al. Anal. Biochem. 48:9-26 (1972); Lowenstein, H., et al.,
25 ibid. 71:204-208 (1976)).

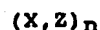
SUMMARY OF THE INVENTION

This invention provides a low cost, novel, solid phase assay of high sensitivity and efficiency for determination of the activity of tyrosine and serine/threonine specific protein
30 kinases in crude enzyme preparations. The assay substrates are gel-immobilized low molecular weight polypeptides including

tyrosine or serine/threonine. For example, an assay for TPK may utilize as a substrate the synthetic peptide (Glu:Tyr 4:1) immobilized as a polyacrylamide gel preferably having microtiter size wells.

- 5 Polypeptides useful as substrates are commercially available and may be synthesized by known techniques.

Preferred substrate polypeptides include those depicted by the schematic formula



- 10 in which X is a tyrosine or serine/threonine residue, Z is any other amino acid residue and the ratio of X to Z is within the range of from about 1:1 to about 10:1, preferably about 4:1 and n is preferably from about 30 to about 100. In the case of serine/threonine kinases, natural proteins such as casein, skim
15 milk, etc. can be used as substrates.

Gels known to the art to be useful for protein kinase assays are useful as a class in the invention. Such gels include, among others, polyacrylamide gels, agar gels, and starch gels. Polyacrylamide gels are preferred.

- 20 The substrate polypeptide may be distributed throughout the gel or localized in the vicinity of wells incorporated into the gel.

- The kinase reaction is initiated in known manner by adding crude enzyme solutions and [γ - 32 P] ATP metal ion mixtures into
25 such wells. Typical phosphorylation reaction mixtures include enzyme and ATP metal ion mixtures, for example a mixture containing 50 mM Tris-HCl buffer, pH 7.4, containing 40 μ M [γ - 32 P] ATP (~12,000 cpm/pmole), 15 mM MgCl₂, and 2 mM MnCl₂. After phosphorylation, the reaction mixtures are removed and
30 the gels are prewashed in water and electrophoresed to completely remove free radioactive ATP. 32 P incorporation into

the immobilized amino acid specific substrate can be detected by autoradiograph and quantitated by liquid scintillation counting of the cut gel.

DESCRIPTION OF THE FIGURES

5 Figure 1. Phosphorylation of immobilized TPK specific substrate by soluble TPKs.

Control and substrate gels were made respectively in the absence and presence of 2 mM (Glu:Tyr 4:1)_n (E⁴,Y¹)_n, wherein "n" is from about 40 to about 100. The wells of the substrate
10 gel contained 30 μ of [γ -³²P] ATP-metal mixture and crude enzyme [40% (NH₄)₂SO₄ supernatant of human placental 100,000 x g cytosolic fraction] (well 3). The wells of the control gel contained [γ -³²P] ATP-metal mixture and the crude enzyme in the presence of 1 mM cold ATP. In each case, the
15 ATP-metal mixture consisted of [γ -³²P] ATP-metal ion mixture of 50 mM Tris-HCl buffer, pH 7.4, containing 40 μM [γ -³²P] ATP (-12,000 cpm/pmole), 15 mM MgCl₂, and 2 mM MnCl₂. The phosphorylation reaction was allowed to proceed for 40 minutes at 25°C and stopped by adding 5 mM of ATP. After the reaction
20 the gel was washed with water and electrophoresed to remove excess [γ -³²P] ATP. The autoradiograms of the control and substrate gels are shown.

Figure 2. Phosphoamino acid analysis of the reaction mixture and the washed gel.

25 The reaction mixture and the washed gel from well 3 of the substrate gel (Figure 1) were subjected to phosphoamino acid analysis. RM and G represent the analysis of the reaction mixture and the gel, respectively. The standard phosphoamino, phosphothreonine, phosphoserine and phosphotyrosine acids
30 identified by ninhydrin are indicated.

Figure 3. Alkaline treatment of the phosphorylated proteins and TPK substrate. Two substrate gels containing four wells each were prepared, and the TPK reaction was performed as described with reference to the substrate gel of Figure 1.

5 Specifically, the reaction was performed in a final volume of 30 μ l using 40% $(\text{NH}_4)_2\text{SO}_4$ supernatant of the placental 100,000 x g cytosolic fraction. A: The upper panel shows an autoradiogram of the washed gels. The low intensity well in each gel corresponds to the no enzyme control. The lower panel

10 shows autoradiograms of the gels after treatment with 1N KOH (left) and neutral water (right) at 56°C for 2 hours. B: The reaction mixture taken from wells in A were analyzed by SDS-PAGE (7.5% gel). The fixed gels were autoradiographed and were found to display comparable intensity. The figure shows

15 an autoradiogram of the gel strips after a 56°C, 2 hour treatment in 1 N KOH (left) and neutral water (right). During the treatment at 56°C, for 2 hours, the solid phase gels excised from A were coincubated with the respective strips. The blots at the bottom of the strips correspond to the

20 autoradiograph of these solid phase gel pieces.

Figure 4. Solid phase method application for TPK preparations partially purified from placental 100,000 x g cytosolic fractions.

Control and substrate gels were prepared and samples were

25 subjected to phosphorylation reaction in the manner described with reference to Figure 1. The numbered wells contained: no enzyme (1 & 2); DE52 peak-I, 25 μ l (3 & 4); DE52 peak II, 25 μ l (5 & 6); heat-denatured DE52 peak II, 25 μ l (7 & 8); DE52 peak-I, 1 μ l (9), 5 μ l (10), 10 μ l (11), and 15 μ l (12);

30 $(\text{E}^4, \text{Y}^1)\text{n-Sephrose}$ column fractions, 25 μ l each of #2 (13), #4 (14), #6 (15), #8 (16), #10 (17), and #11 (18); blank

(19 & 20). A shows autoradiograms of the control and substrate gels before (upper panel) and after (lower panel) washing. B shows details of the results.

Figure 5. Correlation between enzyme concentration and ^{32}P incorporation into the immobilized TPK substrate.

A substrate gel was prepared and samples were subjected to phosphorylation reaction in the manner described with reference to Figure 1.

Shown is the autoradiogram of the substrate gel containing: blank (1); no enzyme (2-4); DE52 peak-II, 5 μl (5-7); 10 μl (8-10); and 20 μl (11-16). The wells 14-16 contained cold 4 mM ATP.

Figure 6. Two tier gels.

A gel with 16 wells was prepared in the absence of TPK substrates. Into the wells, 100 μl of the acrylamide solution containing various concentrations of $(\text{E}^4, \text{Y}^1)_n$ were added to prepare upper substrate gels; 0 mM (1), 0.05 mM (2), 0.1 mM (3 & 4), 0.2 mM (5 & 6), 0.5 mM (7 & 8), 0.75 mM (9 & 10), 1.0 mM (11 & 12), and 2.0 mM (13-16). An equal amount of the placental 100,000 x g cytosolic fraction was added to all the wells except well 15 and 16 which contained butter.

Phosphorylation reactions were started by the addition of ATP-metal ion mixture and proceeded thereafter as described in reference to Figure 1. The autoradiogram of the gel is shown.

25 EXAMPLE I--METHODOLOGY

Acrylamide/Bis solution containing 2 mM $(\text{E}^4, \text{Y}^1)_n$ was allowed to form 7.5% gels according to Davis (Davis, J., Ann. N.Y. Acad. Sci. 121:404-427 (1964)). Generally, 15 ml of the gel solutions were poured into bottle caps, yielding 2 gels with a diameter of 5 cm and a thickness of 4 mm. Microtiter plates with 96 wells were used as molds to make wells in the

polyacrylamide gels. The polymerized gel was kept at 25°C for -3 hours and overnight at 4°C. With gels still in their casts (bottle caps), 30 μ l of phosphorylation reaction mixture, as described in the Summary of the Invention or as indicated in the description of the figures, containing crude enzyme preparations from human placental 100,000 x g cytosolic fraction, 40 μ M [γ -³²P] ATP (~12,000 cpm/pmole), 15 mM MgCl₂, and 2 mM MnCl₂, were added to the wells. Bromphenol blue (0.001%) was included in the reaction mixtures to facilitate delivery of the samples into wells. Control wells included the reaction mixture without enzyme. Reactions were allowed to continue at 25°C for 40 minutes with gentle shaking and were terminated by adding 5 mM ATP. After removal of the reaction mixtures from the wells, gels in the casts (bottle caps) were washed for 10 minutes 4 times with 1 liter stirring water in an upside down orientation. Each gel was taken out of its cast and placed on the flat platform of a horizontal gel electrophoresis apparatus (e.g. BRL Model H5). The wells in the gel were filled with 50 μ l of Laemmli's sample buffer (See, Laemmli, U.K. Nature (London) 227:680-685 (1970)). The gel was then electrophoresed in 25 mM Tris-glycine buffer, pH 8.3, containing 0.1% SDS. The gel was then fixed in 50% methanol/10% acetic acid for 1 hour and subjected to autoradiography. Quantitative data were obtained by cutting gel wells and counting them for radioactivity with a liquid scintillation counter.

The supernatant from the reaction mixtures was mixed with bovine serum albumin (400 μ g/ml) and the proteins precipitated with cold trichloroacetic acid (15%). The resulting pellet was washed in acetone and dried in vacuo. The washed gel well was dried by evaporation and the residual mass was transferred to

an ampoule. 6N HCl containing standard phosphoamino acids, i.e., phosphothreonine, phosphoserine, and phosphotyrosine, was added to the samples and the ampoule sealed in vacuo. Partial hydrolysis was done at 105°C for 2 hours. The residual shrunk gel was removed and the supernatant evaporated. The concentrated samples were subjected to paper electrophoresis (pH 3.5, 1.5kv, 45mA, 2h). The standard phosphoamino acids were identified by ninhydrin and the radiolabeled phosphoamino acids were visualized by autoradiography.

10

EXAMPLE II

Solid phase TPK assay in presence of Ser/Thr kinases and their substrates: 40% (NH₄)₂SO₄ supernatant of human placental 100,000 x g cytosolic fraction which is rich in endogenous Ser/Thr phosphorylation activity was used to demonstrate that the solid phase TPK assay is not affected by Ser/Thr phosphorylation interference.

Two polyacrylamide reaction gels were prepared as described in Example I in the absence (control gel) or presence (substrate gel) of (E⁴,Y¹)n. Four wells contained [γ -³²P] ATP-metal ion mixture (well 1), [γ -³²P] ATP-metal ion mixture together with excess cold ATP (well 2), [γ -³²P] ATP and crude enzyme (well 3), and [γ -³²P] ATP, crude enzyme and excess cold ATP (well 4).

Autoradiograms of the two gels are shown in Figure 1. Only the well containing crude enzyme (well 3) in the substrate gel showed a distinct signal while well 3 in the control gel does not show any significant background noise. The phosphopolypeptide signal is abolished when excess cold ATP is added (well 4 vs. well 3 in the substrate gel).

When the reaction mixture from this assay was subjected to phosphoamino acid analysis, phosphoserine and phosphothreonine were seen as major components in addition to phosphotyrosine

(Figure 2, lane RM), which indicates a significant level of endogenous Ser/Thr phosphorylation. However the washed substrate gel demonstrated the presence of only phosphotyrosine (Figure 2, lane G). This demonstrates utility of the solid phase TPK assay to measure tyrosine phosphorylation without interference from Ser/Thr phosphorylation.

EXAMPLE III

Alkaline stability of the phosphorylation measured in the solid phase TPK assay: To further determine that the solid phase TPK assay is monitoring tyrosine phosphorylation, the phosphorylated substrates in the gel described in Example II were subjected to alkaline treatment. Two mini-gels with four wells each were used to perform the TPK assay in triplicate. One well in each gel held the control with no enzyme. The gels shown on the top half of Figure 3A were subjected to treatment with alkaline (1N KOH) or neutral water at 56°C for 2 hours. Autoradiograms of the treated gels are shown in the lower half of Figure 3A. While the heat treatment seems to have eliminated the background noise, the signals remained unaffected after alkaline treatment.

To confirm that the alkaline treatment conditions used can discriminate phosphate esters, the reaction mixtures from the solid phase wells were electrophoresed in duplicate on a 7.5% SDS-PAGE. Figure 3B shows that the majority of endogenous phosphorylation was alkali labile. This suggests the occurrence of aliphatic phosphate groups in the phosphoproteins of the reaction mixture. One well each from Figure 3A was included as an internal control of the experiment described in Figure 3B. While alkaline treatment largely abolishes endogenous protein phosphorylation, it has no influence on the phosphorylation that occurs on the immobilized tyrosine

specific exogenous substrate in the gel. These results demonstrate that the solid phase TPK assay offers specificity of phosphorylation at tyrosine residues.

EXAMPLE IV

5 Measurement of TPK activity in column fractions: This example demonstrates the practicality of the solid phase assay. Figure 4A shows autoradiograms of control and substrate gels with up to 20 wells were prepared in the manner described in the preceding examples. After phosphorylation, the gels
10 were extremely radioactive (Figure 4A, upper panel). The radioactivity due to free [γ - ^{32}P] ATP was efficiently removed by electrophoresis revealing clear signals of ^{32}P incorporation into the immobilized substrate (Figure 4B, lower panel).

 The 100,000 x g cytosolic fraction from human placenta was
15 subjected to DEAE-Cellulose (DE52) and (E^4, Y^1)n-Sephadex affinity chromatography. Fractions were tested for TPK activity by solid phase assays. As shown in Figure 5B, the pooled fractions from DEAE-cellulose chromatography (wells 3-6) showed signals in the solid phase TPK assay. The signal
20 intensity was lowered to background levels when heat-inactivated enzyme was used (wells 7 & 8). Wells 9-12 demonstrate increasing signal intensity as a function of enzyme concentration. As is evident from wells 13-18, the flow-through fraction of (E^4, Y^1)n-Sephadex (fractions 2-6) had
25 no TPK activity (wells 13-15) while the bound protein fractions (fractions 8-11) demonstrate the presence of TPK activity (wells 16-18).

 Because of the proximity of wells and the porous nature of the gel between the wells, it was important to confirm that
30 enzymes do not cross contaminate the wells. As shown in Figure 4B, the wells without enzyme activity (1, 2, 7, 8, 19,

and 20) show no sign of contamination although these control wells are surrounded by enzyme containing wells. Thus in the reasonable time of normal enzyme assays, cross contamination by diffusion across wells is highly unlikely.

5

EXAMPLE V

Quantitative determination of TPK activity by the solid phase TPK assay: TPK activity of crude enzyme preparations at different concentrations was measured in triplicate by the solid phase method. Figure 5 shows the autoradiogram of the substrate gel containing: blank (1); no enzyme (2-4); DE52 peak II, 5 μ l (5-7); 10 μ l (8-10); and 20 μ l (11-16). Wells 14-16 contained excess cold ATP. The wells were excised and the radioactivity was measured by liquid scintillation counting: Well (1), 110 cpm; wells (2-4), 413 \pm 57 cpm; wells (5-7), 646 \pm 78 cpm; wells (8-10), 838 \pm 18 cpm; wells (11-13), 1563 \pm 38 cpm; wells (14-16), 650 \pm 123 cpm. These data indicate that under this condition, 10 μ l of the enzyme preparation or more provide satisfactory signal/noise ratios and reproducible signal values.

20

EXAMPLE VI

Two tier gels: Wells in gels formed in the presence of substrate all have a uniform substrate concentration. Furthermore, a considerable gel mass which lies outside the well area consumes more substrate than actually needed. To overcome this problem, gel solutions containing a variable concentration of $(E^4, Y^1)_n$ were overlayed in the wells made in a 7.5% acrylamide gel. Figure 6 shows the result of experiments in which progressive increases in signal intensity were visible as the substrate concentration was increased from zero to 2 mM.

30

Although the invention is exemplified by assays for TPK, it is readily applied to provide assays for serine/threonine kinase.

I CLAIM:

- 1 1. A solid phase tyrosine or serine/threonine specific
2 protein kinase assay comprising:
 - 3 immobilizing a substrate for said kinase in a gel
 - 4 said substrate containing a polypeptide including
 - 5 tyrosine or serine/threonine residues;
 - 6 placing a sample to be assayed in contact with said
 - 7 gel having said substrate immobilized therein;
 - 8 subjecting said gel while in contact with said sample
 - 9 to phosphorylation; and
 - 10 detecting the degree of phosphorylation of the
 - 11 substrate immobilized in said gel.
- 1 2. The assay of claim 1 in which the phosphorylation is
2 conducted with a reagent which includes a label which can be
3 detected in the substrate upon completion of the
4 phosphorylation reaction.
- 1 3. The assay of claim 2 in which the label is a
2 radioactive isotope.
- 1 4. The assay of claim 3 in which the isotope is ^{32}P .
- 1 5. The assay of claim 1 in which the substrate includes a
2 polypeptide having the schematic formula
$$(X,Z)_n$$
 - 3
 - 4 in which X is a tyrosine or serine/threonine residue and Z is
 - 5 any other amino acid residue and is from about 30 to about 100.
- 1 6. The assay of claim 5 in which the ratio of X to Z is
2 from about 1:1 to about 10:1.
- 1 7. The assay of claim 6 in which the ratio of X to Z is
2 from about 4:1.
- 1 8. The assay of claim 5 in which the substrate polyamide
2 is Glu:Tyr 4:1.

1 9. The assay of claim 5 in which the substrate contains
2 casein or skim milk.

1 10. The assay of claim 1 in which the immobilized
2 polypeptide containing substrate is dispersed throughout said
3 gel.

1 11. The assay of claim 1 in which said gel is provided
2 with a plurality of wells and in which said substrate is
3 immobilized only in the vicinity of at least some of said wells.

1 12. A solid phase tyrosine or serine/threonine specific
2 protein kinase assay comprising:

3 adding a sample to be assayed to a well in a gel
4 containing an immobilized substrate for said kinase;
5 adding phosphorylating reagents to said well to which
6 said sample is added;
7 phosphorylating said substrate in the vicinity of said
8 well;

9 removing the phosphorylation reaction mixture from
10 said well; and

11 detecting the degree of phosphorylation of said
12 substrate.

1 13. The assay of claim 12 in which the assay is a tyrosine
2 specific protein kinase assay.

1 14. The assay of claim 12 in which the assay is a
2 serine/threonine specific assay.

1 15. The assay of claim 12 in which the phosphorylation
2 reagents are labelled.

1 16. The assay of claim 15 in which the label is a
2 radioactive isotope.

1 17. The assay of claim 12 in which the substrate is
2 contained either in the entire gel or only in a portion of the
3 gel inside said well.

- 1 18. The assay of claim 1 or claim 2 in which the gel is a
- 2 polyacrylamide gel, an agar gel or a starch gel.

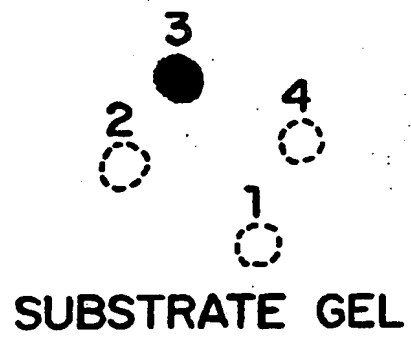
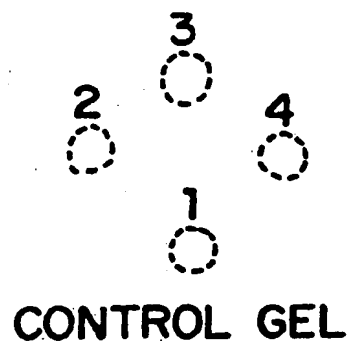


FIGURE 1

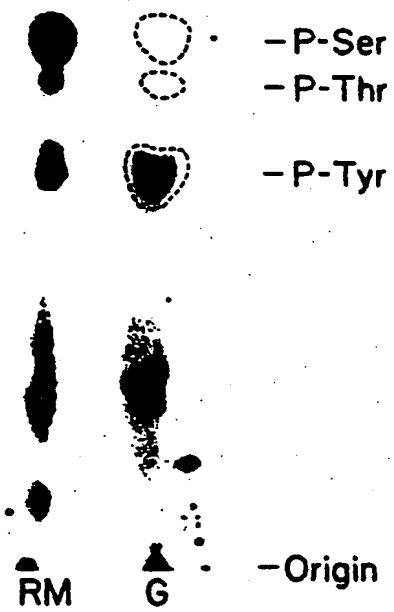


FIGURE 2

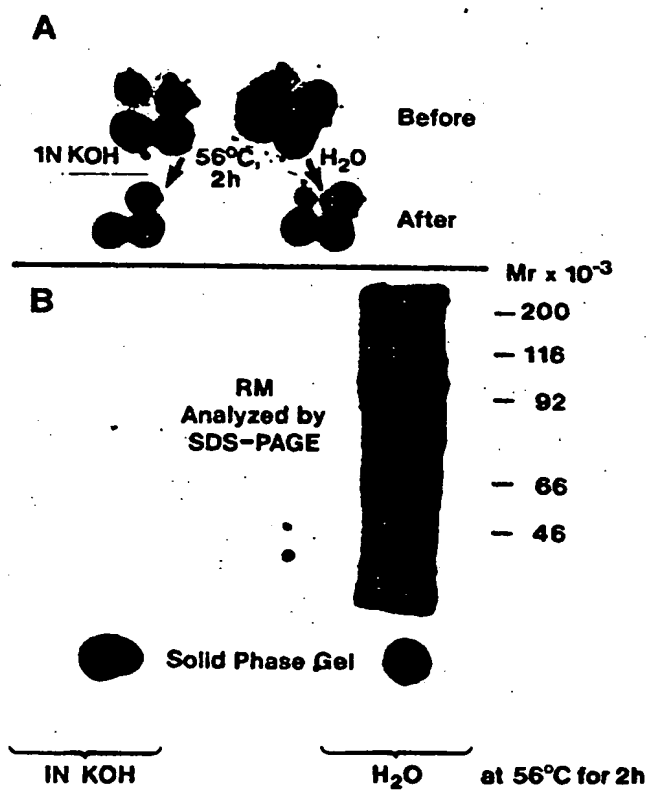


FIGURE 3

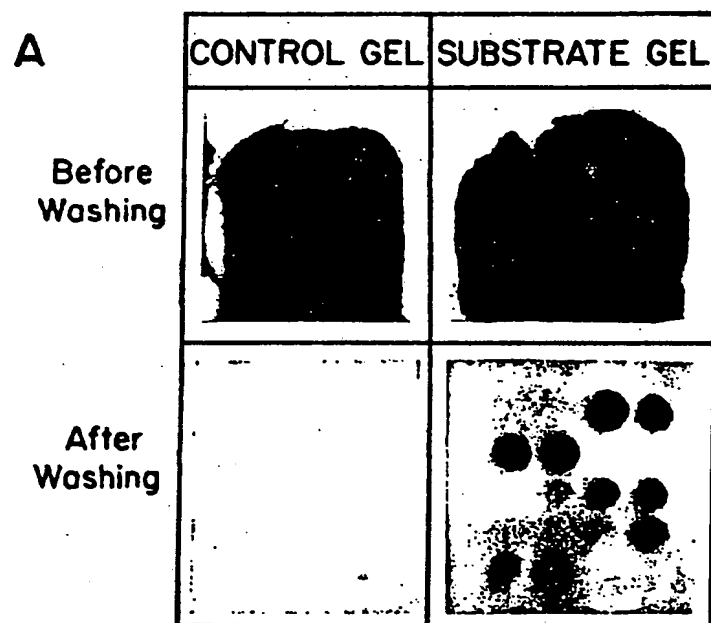


FIGURE 4A

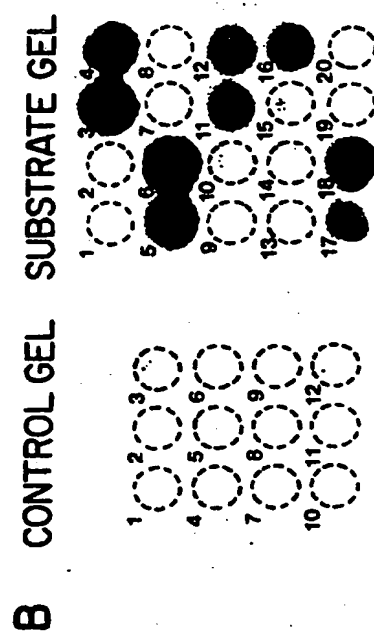


FIGURE 4B

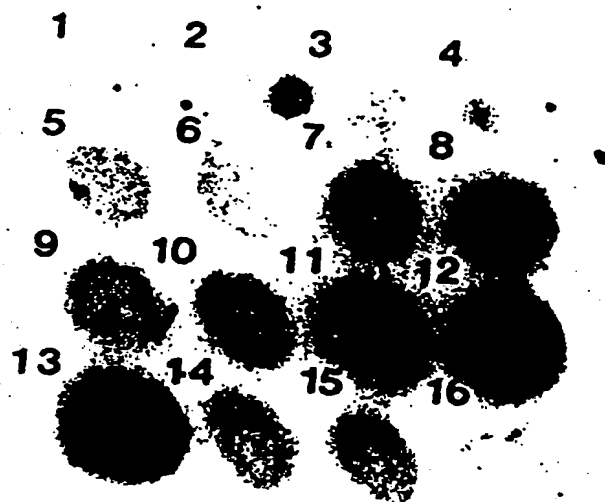


FIGURE 5

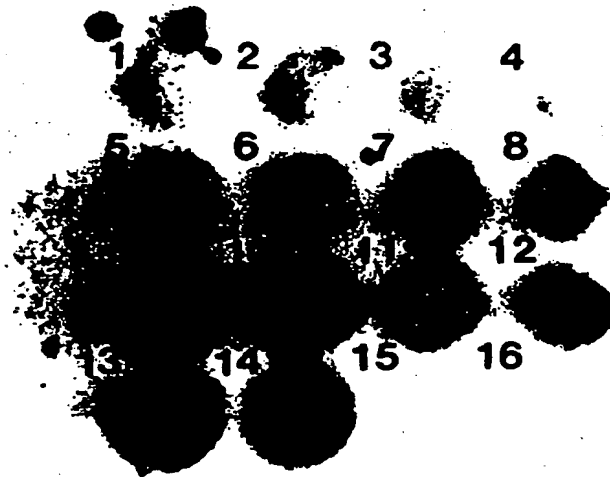


FIGURE 6

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.